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Novel stepwise gradient reversed-phase liquid chromatography separations of humic substances, air particulate humic-like substances and lignins

Milan Hutta*, Róbert Góra

Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina CH-2, 842 15 Bratislava, Slovakia

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Abstract

RP-HPLC using stepwise gradients of dimethylformamide (DMF) in buffered aqueous mobile phase and a wide-pore (30 nm) octadecylsilica column has been applied to the analysis of soil, peat and air particulate humic and humic-like substances (HSs), as well as lignin, in order to demonstrate the usefulness of the approach for their characterization even at trace concentration level. Tandem combination of spectrophotometric (DAD) and fluorimetric detection was used to get more detailed information on chromatographic behaviour of HSs. The results showed that ten-step gradient can induce distinct features of HSs and lignins. Combination of very good DMF solvating and disaggregating properties for HSs and lignins together with wide pore RP sorbent improves surface interactions of the analytes and suppresses influence of size exclusion effects. Thus it provides reproducibility of characterisation profiles and robustness of the method. Very good reproducibility of retention times (from ±0.12 to ±0.36% RSD), of peaks enforced by the step gradient shape supports well defined characterization and/or fractionation of HSs. Evaluated limit of quantitation ($S/N=10$) of selected soil humic acid working standard using fluorimetric detection (ex.470 nm/em.530 nm) was 9.7 μg/ml. This corresponds to 0.5 μg of the humic acid per injection. Calculated limit of detection ($S/N=3$) was 3.3 μg/ml, what corresponds to 0.15 μg of the humic acid per injection and enabled determination of 0.1% (m/m) humic-like substances in 20 mg of air particulates under defined rules and conditions.

Keywords: Air particulates; Peat; Soil; Humic substances; Lignins; Dimethylformamide

1. Introduction

Methods of liquid chromatography (LC) have found widespread applicability in research of humic substances (HSs). The research of HSs profits from their use for purposes of HSs isolation, fractionation and purification beside chromatographic characterization, profiling and analysis. The importance of separation methods in the chemistry of HSs, including LC methods is currently stressed by a review article of Janoš [1]. Among the chromatographic methods those based on size-exclusion effect (SEC) play the most important role. SEC is used for measurement of data on relative molecular masses

*Corresponding author. Tel.: +421-760-296-307; fax: +421-765-425-360.
E-mail address: hutta@fns.uniba.sk (M. Hutta).

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distribution of HSs. However, the results obtained by size-exclusion chromatography of HSs should be carefully judged and accepted, because they are still controversial. The reason is given by natural tendency of HSs to undergone conformational changes and/or aggregation as a response to changed conditions mainly in their aqueous solutions (their own concentration, ionic strength, pH, quality and concentration of accompanying elements, temperature, dielectric constant etc.).

Surprisingly small in comparison to SEC application in HSs research is impact of RP-HPLC methods. Serve at al. [2] analysed low molecular mass acids and aldehydes obtained by acid hydrolysis (6 *M* HCl) of HSs by RP-HPLC on C_{18} column and aqueous acetic acid–acetonitrile mobile phase.

Hayase and Tsubota [3] studied chromatographic behaviour of HSs in RP systems from the point of view of relations between hydrophobicity or molecular mass and retention for sedimentary fulvic acids (FAs). Blondeau and Kalinowski [4] fractionated HSs by hydrophobic interaction chromatography (HIC) in gradients of decreased concentration of sodium chloride in water and in sodium hydroxide. They concluded that fulvic acids (FAs) exhibited weaker hydrophobic interactions than terrestrial humic acids (HAs) and that sedimentary HAs were more hydrophobic than terrestrial HAs. Hirose et al. [5] concluded that RP systems do not allow an effective fractionation of HSs and their chromatograms do not give any useful structural information. However, certain HSs structure-related analytical signal can be obtained when HPLC is combined with sophisticated diode-array spectrophotometric detection (DAD) [6]. HPLC with fluorescence detection (FLD) using C_{18}-bonded phase was tested for trace analysis of HSs in environmental samples, including coral skeletal matter, sea and river water, soils and plants, by Susic and Boto [7]. This method was later modified and applied for determination of HSs in Al containing solutions from Bayer process by Susic and Armstrong [8]. Two-step gradient of cationic ion pairing reagent on wide-pore (30 nm) polymeric reversed-phase enabled Smith and Warwick [9] to get six peaks separation of groundwater fulvic acids.

Low-molecular-mass substances originated from oxidative degradation of lignin and HSs were analyzed in waters, in effluent from a kraft pulp mill [10], in alcoholic beverages [11] and HSs in water samples [12–14] by RP-HPLC. Lombardi et al. [15] developed RP-HPLC method on conventional C_{18} sorbent and stepwise gradient of acetonitrile in aqueous sodium acetate for fractionation of marine organic matter into six fractions detected by UV detection. Later this method was applied to the characterization of HPLC fractionated marine organic matter [16] and marine and terrestrial organic matter [17] by fluorescence spectra

Woelki et al. [18] used C_{18} columns of varying pore diameter (10, 100 and 400 nm) and showed that maximum recovery was ensured at 100 nm pore diameter for various HSs. They attributed hydrophobicity change of HSs to disruption of the tertiary structure. Step-wise gradient elution (three steps of 100% water, 30% acetonitrile in water and 100% acetonitrile) allowed them to obtain five characteristic peaks of HSs. The same gradient shape of methanol gave recoveries of 45% instead of 100% recovery gained in the acetonitrile gradient.

Fluorescence detection proved to be valuable tool for simultaneous determination of concentration and molecular mass of HSs in river waters [19]. Problems with aggregation of HSs in solutions and mobile phases were revealed by recent studies of Preusse et al. [20]. They observed that the retention and fractionation of HSs depend significantly on the amount of injected sample.

Wu et al. [21] fractionated fulvic acids by immobilized metal ion (CuII) affinity chromatography with acidic and chelating eluents. The fractions were characterized by gradient RP-HPLC on C_{18} column and by SEC.

In majority of the chromatographic studies of retention behavior of HSs in RP-HPLC phase systems [2,4–8,10–17,19–21] RP sorbents with pore diameters of 6–12 nm were used, so the results were probably influenced also by the size exclusion phenomena, because estimated exclusion limit for typical bare silica having 10 nm average pore diameter is close to relative molecular mass 5000 [22]. RP materials have the value even slightly lower due to covalently bonded functional groups filling part of the pore. Mobile phases used in the above cited articles are typical ones for RP-HPLC of small molecules, they are usually prepared of water, methanol, acetonitrile, or tetrahydrofuran, pH is buffered or surprisingly frequently non-buffered.

Our intention was to use in gradient RP-HPLC

good solvent for polymeric, polyelectrolyte and composed organo-inorganic substances, because all of these features are attributed to HSs. The strict control of pH by buffer having sufficient required buffering capacity we consider as one of the important prerequisites of robust RP-HPLC method for HSs analysis, their characterization and/or fractionation.

Excellent ability of dimethylformamide (DMF) beside the other good solvents as formamide, dimethylsulfoxide and dioxan to dissolve HSs from soil giving dark brown solution was recognized very early [23]. DMF has been termed universal organic solvent [24] strongly hydrogen-bonded with high solubility parameter $\delta = 12.1$ and slow rate of evaporation [25]. DMF is miscible with water in all proportions and is useful as a solvent for electrolytes, beside solubilization of miscellaneous organic materials—including some polymers and plastics. It dissolves also various inorganic salts and materials (including iron, calcium, magnesium salts up to concentration 20% (m/m)). It forms complexes with numerous Lewis acids. UV cut-off value of DMF is slightly below 280 nm [26].

Actual density data for Suwannee River fulvic acid (SRFA) has been obtained in a variety of solvent systems, among others THF, DMF and DMSO. THF by itself or in conjunction with water or HCl is not capable completely disaggregate SRFA. Aggregation of fulvic acid molecules in aqueous solutions is apparent at all concentrations in excess of 0.1% (m/m). DMSO is judged to be the best solvent system for disrupting hydrogen bonding in SRFA in the presence of small amounts of acid and DMF is only slightly less effective [27].

DMF is also good solvent for SEC of lignins and lignocarbohydrates complexes [28,29], according to recently accepted theory precursors of terrestrial HSs. Its use in HPLC is restricted [30]; Ryba [31] evaluated DMF in concentration range not exceeding 10% as a mobile phase component in RP-HPLC of weakly basic substances on C_{18} column for suppression of influence of residual silanols to retention and peak tailing.

Beside old and notorious interest in behavior and structure elucidation of water HSs, including sedimentary HSs and terrestrial (soil) HSs, new focus is evident in analysis, characterization and fractionation of humic-like substances in airborne particulate matter [32,33] and water soluble organic matter from atmospheric aerosol [34] using solid-phase extraction procedures in RP mode.

2. Experimental

2.1. Instrumentation

Study of the retention behaviour and evaluation of spectral characteristics of the selected groups of humic substances was carried out by the HPLC system LaChrom (Merck-Hitachi, Darmstadt, Germany) consisting of pump L-7100 provided by a quaternary low-pressure gradient, autosampler L-7200, column oven L-7300, diode-array detector L-7450A, fluorescence detector L-7480, interface D-7000, PC data station with software HSM ver. 3.1 and on-line four channel solvent degasser L-7612. Measured dwell volume of the system including column was 3.80 ml and should be considered when gradient mixing profile and chromatogram appearance is to be compared.

Extractions of humic substances from soil, peat and air dust particulates were done with the aid of shaker KS 125 (IKA Labortechnik, Junke and Kunkel, Germany). Digital pH meter Radelkis OP-211/1 (Budapest, Hungary) provided by combined glass/AgCl electrode was used for mobile phase pH measurement.

Partisol 2000-H air sampler (Ruprecht&Patashnik, Albany, NY, USA) certified for the air particulates collection on filters and air contaminants on polyurethane foam (PUF) was used. The measured air flow-rate is corrected with respect to the air pressure gradient across the filter and the PUF cartridge, respectively.

2.2. Methods

Separation was carried out using a LiChroCART column 250×4 mm filled by wide pore octadecylsilica LiChrospher WP 300 RP-18, 5 μm spherical particles, guarded by LiChroCART 4×4 mm pre-column filled by LiChrospher WP 300 RP-18, 5 μm particles. Dead volume (calculated from the manufacturers data [37]) of the column was 2.55 ml and is equal to the retention volume of the first

HSs eluted peak. Flow-rate was maintained at 0.50 and 1.00 ml/min, respectively.

Separation conditions for optimised gradient elution of humic substances were as follows. Mobile phase A composition was: aqueous phosphate buffer (pH 3.00, 50 m*M*) containing 1% (v/v) dimethylformamide (DMF). Mobile phase B was 100% DMF.

2.2.1. Linear gradient

Gradient program was set from 0.0 to 5.0 min isocratic 0% B in A, from 5.1 to 35.0 min linear increase from 0% B in A to 95% B in A, from 35.1 to 40.0 min isocratic 95% B in A, from 40.1 to 45.0 min linear decrease from 95% B in A to 0% B in A (see Fig. 1) and between runs 10 min reequilibration.

2.2.2. Stepwise gradient

Gradient program was set from 0.0 to 3.6 min isocratic 0% B in A, and from 3.7 min every 4 min there was isocratic step added increasing content of B in A by 10% up to the last step increased by 9% ending in 99% B in A, maintained till 55.0 min isocratic 99% B in A (see Fig. 2) from 55.1 to 60.0 min linear decrease from 99% B in A to 0% B in A and between runs 10 min reequilibration was maintained. The same gradient program was evaluated for both 0.50 and 1.00 ml/min, respectively (see Fig. 3).

Column oven temperature was maintained at 35.0±0.1 °C. Injection volume of 50 μl was injected by the autosampler. Wavelength range of DAD was set to 280–800 nm. Monitoring wavelength was set to 420 nm. Fluorescence detection parameters were set to excitation wavelength 470 nm and emission wavelength 530 nm according to published data [23] on fluorescence spectra measured in DMF solutions, photomultiplier gain medium was chosen.

2.3. Materials

Target group of humic substances was obtained by the procedures published by Kandráč et al. [35], Procházková et al. [36] and Góra [51]. Commercial humic acids were purchased from Aldrich and Fluka.

Solutions of humic substances were prepared daily fresh by dissolution of weighed HSs at 3 mg/ml concentration level in 0.05 *M* NaOH. Short description of analysed HSs and exact concentrations of individual HSs are given in Table 1.

Solution of alkalilignin of wheat straw was kindly provided by Fellegi-Techneco, Bratislava, Slovakia.

Air particulates (locality Topolove, Bratislava—field with arable soil) were collected at the quartz

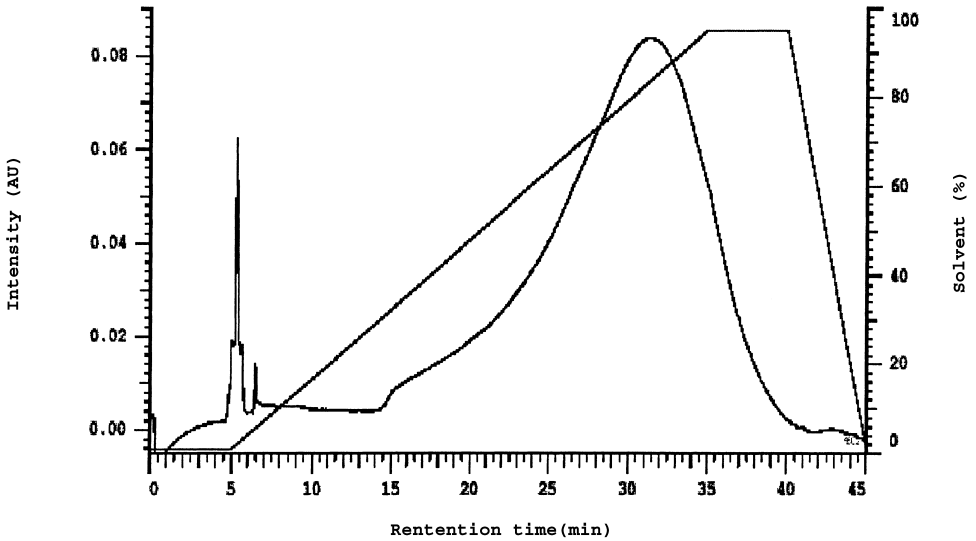

Fig. 1. Chromatogram of peat humic acid HFA PE obtained by a linear gradient elution. Flow rate was set to 0.5 ml/min. Spectrophotometric detection (DAD) at monitoring wavelength 420 nm. For the other conditions, see Section 2.

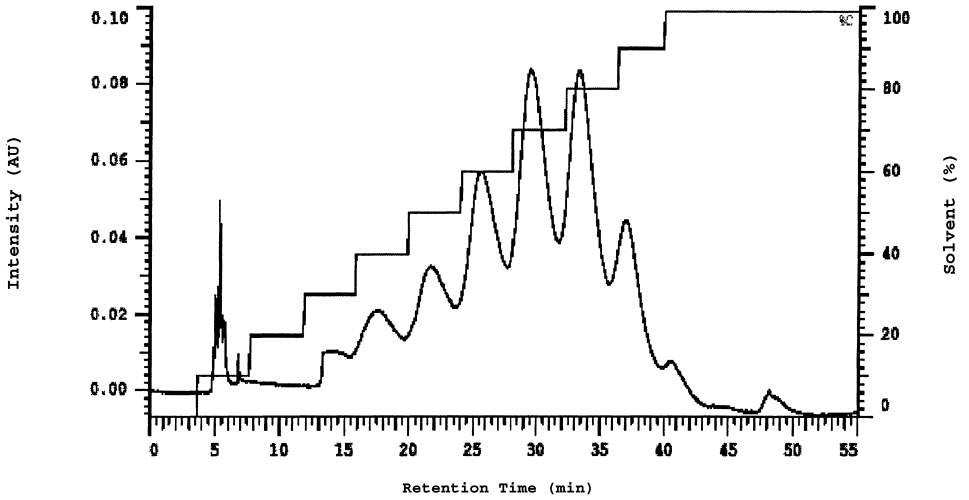

Fig. 2. Chromatogram of peat humic acid HFA PE obtained by a stepwise gradient elution. Flow-rate was se to 0.5 ml/min. Spectrophotometric detection (DAD) was done at monitoring wavelength 420 nm. For the other conditions, see Section 2.

microfiber filter OM-A for air sampling (diameter 47 mm, Whatman) with the aid of Partisol 2000-H air sampler (Ruprecht&Patashnik, Albany, NY, USA). Sampled air volume was 110 m^3, air flow-rate 1.0 m^3/h). Mass of sampled particulates was determined gravimetrically and corrected to moisture. Weight of the particulate matter was 81.0 mg. One fourth of the filter (cross-cut and corresponding to 20.25 mg of the particulates) was extracted successively twice by 1 ml of 0.05 *M* NaOH in water for 2 h using laboratory shaker, so that 2 ml of the extract were obtained. The solution was then filtered through the

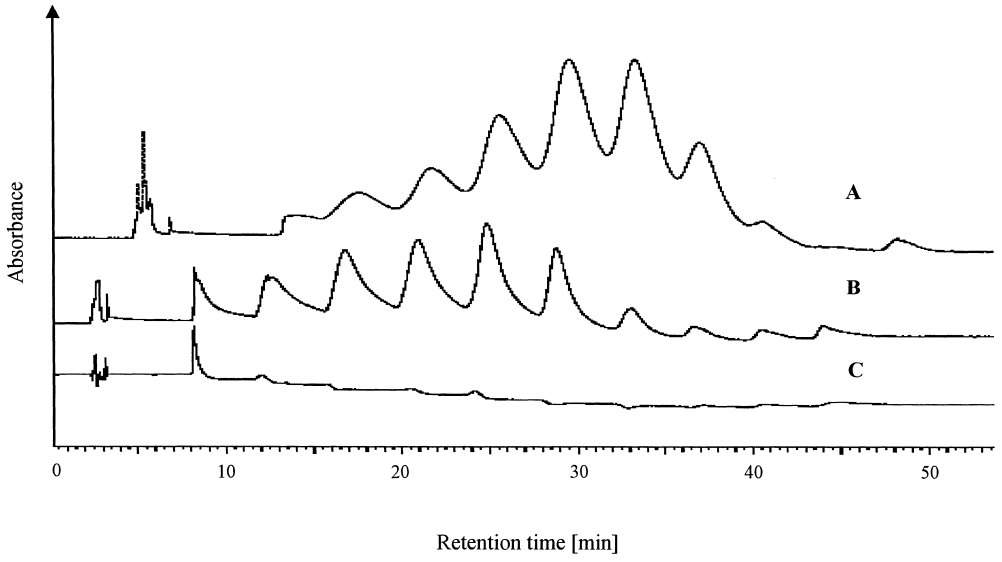

Fig. 3. Effect of flow-rate on separation resolution of peat humic acid HFA PE obtained by the stepwise gradient elution. Chromatogram A: flow-rate was set to 0.50 ml/min. Chromatogram B: flow-rate was set to 1.00 ml/min. Chromatogram C: blank run at flow-rate 1.00 ml/min. Measured at 420 nm. For the other conditions, see Section 2.

Table 1
Selected data of the analysed HSs

Humic substance abbreviation	Source	Relative molecular mass	Injected concentration of HSs [mg/ml]
HA Al	Aldrich	500–1000[a]	3.25
HA Fl	Fluka	1200[a]	2.85
HA Z	Isolated from soil, locality Dunajská Streda [35,36]	10 000–21 000 (SEC, Sephadex G-75) [51]	2.97
HA M	Isolated from soil, locality Dunajská Streda [35,36]	10 000–21 000 (SEC, Sephadex G-75) [51]	2.99
HFA PE	Isolated from peat, locality Suchá hora [51]	8000–10 000 (SEC, Sephadex G-75) [51]	3.20
APHLS B2	Isolated from air particulates, locality Bratislava (see Section 2)	–	≈0.1

[a] Information obtained from the product catalogue. All mentioned localities are in Slovakia.

same kind filter (previously purified by 0.05 *M* NaOH) denoted as APHLS B2 and analysed in the HPLC system.

All humic substances were separated using LiChroCART column 250×4 mm filled by a wide pore octadecylsilica LiChrospher WP 300 RP-18, 5 μm spherical particles and guarded by LiChroCART 4× 4 mm pre-column filled by LiChrospher WP 300 RP-18, 5 μm (Merck, Darmstadt, Germany). The column is devised mainly for fast reversed-phase analysis and preparation of peptides and proteins with the relative molecular mass up to 20 000 and tRNA molecules [37] without size exclusion effects. The pore size of 30 nm allows an efficient mass transfer and a very good recovery, particularly of strong hydrophobic peptides. Its specific surface area is 80 m^2/g and pH stability is declared to be within the range 2.0–7.5.

Dimethylformamide (Merck, Darmstadt, Germany, or Lachema, Brno, Czech Republic) was of pro analysi purity. Water for gradient HPLC was prepared by Labconco Pro-PS unit (Labconco, Kansas City, KS, USA). Phosphoric acid (Lachema, Brno, Czech Republic) and sodium hydroxide (Merck, Darmstadt, Germany) of pro analysi grade were used for preparation of buffered mobile phases.

3. Results and discussion

3.1. Optimisation of separation conditions

Initial careful attempts to characterize HSs by a linear gradients of DMF were done in non-buffered mobile phases and the choice was influenced by available information on potential hydrolysis of DMF under acidic conditions [25] and potential swelling or dissolution of plastic materials, in our case the pump and injector seals. However, initial runs in non-buffered or pH 7 buffered mobile phases revealed, that under these conditions reproducibility of analyses is poor and the apex of elution profile of HSs is shifted to low retention times. Analyses done with the phosphate buffered mobile phases of successively decreased pH have clearly shown the tendency of shift of the signal maximum of HSs chromatographic profile toward higher retention times. The best result as is shown at Fig. 1 by example of peat humic and fulvic acids HFA PE was obtained using aqueous phosphate buffer (50 m*M*) at pH 3.00 as weaker mobile phase component. The peak maximum is eluted by mobile phase containing 63% (v/v) of DMF in the buffer. Similar profiles having the shape of broad peak were measured also for the other

HSs and even for lignin sample. First sharp peak elutes in dead time of the column (t_o=5.1 min at 0.5 ml/min), or is slightly delayed and can be attributed to free hydrophilic components of HSs and/or hydrophilic components released by DMF solvation of HSs. We suppose that exclusion effects (total exclusion) are not probable, because the range of relative molecular masses of restricted inclusion of the analytes for 30 nm pore diameter is relatively broad [22], ranging approximately from $4\cdot10^4$ to $2\cdot10^6$. Peptide or protein molecules with relative molecular mass around $2\cdot10^4$ have not restricted access into the pores [37]. The broad peak itself starts to be eluted by 19% (v/v) of DMF in the buffer and is completely eluted by 95% (v/v) of DMF. The above values are recalculated with respect to the dwell volume of the chromatographic system.

The shape of chromatogram is featureless and HSs fractionation reproducibility in this case could be based only on reproducibility of the profile.

In the next step we focused to optimisation of such gradient shape which could enforce rugged features of HSs profiles in a similar fashion as is created in capillary isotachophoresis (CITP) of humic acids by use of spacers published by Kopáček et al. [38] and Nagyová and Kaniansky [39]. Stepwise gradient was optimised on the basis of the data gathered during the linear gradient profile optimisation and enabled us to extract and emphasize characteristic features (in the form of narrower peaks) out of the broad peak obtained by the linear gradient (Fig. 2). This figure shows ten-step gradient shape as an output signal of the pump beside chromatographic record of the same peat humic and fulvic acids HFA PE (compare with Fig. 1). In comparison to the depicted step gradient program profile actual step gradient is shifted by 7.60 min (at flow-rate 0.5 ml/min) and its first step is observed at 11.20 min (see also Fig. 3A). Except the first step, start of every next gradient step overlaps with the valley between peaks. Thus, the two highest peaks are eluted by 50 and 60% (v/v) of DMF in the aqueous phosphate buffer with pH 3.00 regardless the flow-rate. As is apparent this 10 step gradient provides good tool for exact fractionation and characterization of HSs.

Further attempt to increase resolution of the chromatographic peaks separation was done via change of the mobile phase flow-rate, which was doubled to 1.00 ml/min (Fig. 3). At this flow-rate passage of the first gradient step through the detector cell is seen as a refraction error peak at 7.5 min on a blank chromatogram (Fig. 3C) and chromatogram of HFA PE, respectively. The other steps are visible as small and relatively narrow peaks in comparison to HSs peaks (compare Fig. 3C and B). Observed result seems to be controversial from the point of view of chromatographic theory, but does not. The improvement of separation resolution (Fig. 3A vs. B) results from doubling of the mobile phase volume in each of the gradient steps eluting the fractions of the humic substances at doubled flow-rate. Further increase of resolution was however hindered by excessive pressure drop.

Apparently, separation principle is based on hydrophobic interactions in used reversed-phase system. In aqueous solution at pH 3 humic acids (HAs) are close to their precipitation. Their carboxylic functional groups are in a protonated form stabilised mainly by intramolecular hydrogen bonds. Increasing concentration of DMF solvates many kinds of functional groups present in HAs, or more generally HSs [25]. In the case of DMF and HS we could achieve also specific interaction, based on specific hydrogen bonding between peptide-like $O{=}C{-}N\lessgtr$ structural feature of certain molecules (known e.g. in peptides, pyrrolidones, and also DMF) and phenolic hydroxyl (–OH) or aromatic carboxyl (–COOH) or aldehyde (–CHO) functional groups of HSs and lignins. This type of interaction is responsible e.g. for decrease of effective mobilities of aromatic acids [47] or humic acids [48] in the presence of soluble polyvinylpyrrolidone (PVP) in a CITP electrolytes and is effectively used for characterization and fractionation of humified compounds on a solid PVP (polyvinylpolypyrrolidone PVPP) [49,50]. However, this hypothesis must be proved and together with possible influence of this structural feature of DMF on disaggregation of HSs aggregates is under investigation.

At this point it should be mentioned that the mobile phase flow-rate must be selected carefully, because binary mixtures of DMF–water show viscosity maximum at around 35 mol% DMF in water with viscosity 2.55 mPa·s (water viscosity at 20 °C is 1.00 mPa·s, water viscosity at 25 °C is 0.8937 mPa·s, DMF viscosity at 25 °C is 0.80 mPa·s). Thus, we

observed at the flow-rate 0.50 ml/min (1.00 ml/min, alternatively) relatively complex pressure profile with increase of pressure from 4.4 MPa (9.0 MPa) in initial mobile phase to 11.6 MPa (2.30 MPa) in 50% (v/v) DMF and 60% (v/v) DMF, then decrease to 5.0 MPa (12 MPa) in 99% (v/v) DMF followed by 13.0 MPa (23.5 MPa) pressure pulse as a result of fast down-slope gradient ending at 4.1 MPa (9.0 MPa) in the initial mobile phase composition. Pressure profile served as one of system suitability tests and we never observed clogging of the system by precipitated HSs.

During our experimental work we did around 300 RP runs and together with SEC experiments more than 10 l of DMF flowed through the system without damage of the seals. Hydrolysis of DMF to dimethylamine and formic acid was not critical parameter under the selected conditions, maybe due to the fact that contact time of DMF and mildly acidic phosphate buffer was relatively short during the gradient elution.

3.2. Profiling and characterisation of HSs

The devised stepwise gradient chromatographic method with tandem DAD and fluorimetric detection was used for characterization of HSs by their chromatographic profiles. Fig. 4 shows typical examples of the profiles as resulted from analysis of humic substances of various origin. From the drawing is evident that the chromatographic method is capable to distinguish among the humic substances, here in majority humic acids. Moreover, alkalilignin profile can be resolved from the HSs profiles. The most hydrophilic seems to be mixture of peat humic and fulvic acid (Figs. 4 and 5, chromatogram HFA PE). This humic and fulvic acid mixture was obtained by sequential neutralization of aqueous peat slurry by addition of solid NaOH to pH 7.00, ultrafiltration of the solution and water evaporation. In contrary, the most hydrophobic is alkalilignin, which was added to the set to show the differences between the two significantly different groups of substances. Chromatograms of soil humic acids measured at 420 nm (Fig. 4, chromatograms HA Al, HA Z and HA FL) look quite similar at first sight, but proportions of individual peak heights and areas differ. Humic acid HA M profile is evidently different. Fluorescence detection gives chromatograms (Fig. 5) with much distinctive features enabling to differentiate among the HSs. Fluka humic acid HA FL was probably obtained by simple NaOH extraction of soil and precipitation by HCl, what probably results in small peaks superimposed to the HA profile. Low molecular mass substances were removed during the preparation and purification of HA M and HA Z by

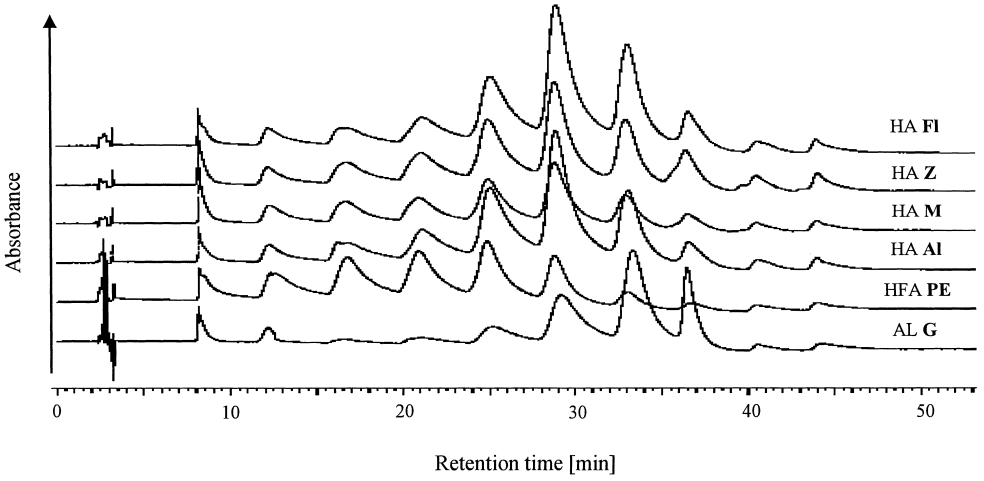

Fig. 4. Chromatographic profiles of various humic substances and alkalilignin as obtained by stepwise gradient elution at 1.00 ml/min and detected at 420 nm monitoring wavelength. Abbreviations: AL G, alkalilignin; HFA PE, peat humic acid; HA Al, Aldrich humic acid; HA M, soil humic acid; HA Z, soil humic acid; HA Fl, Fluka humic acid. For the other conditions, see Section 2.

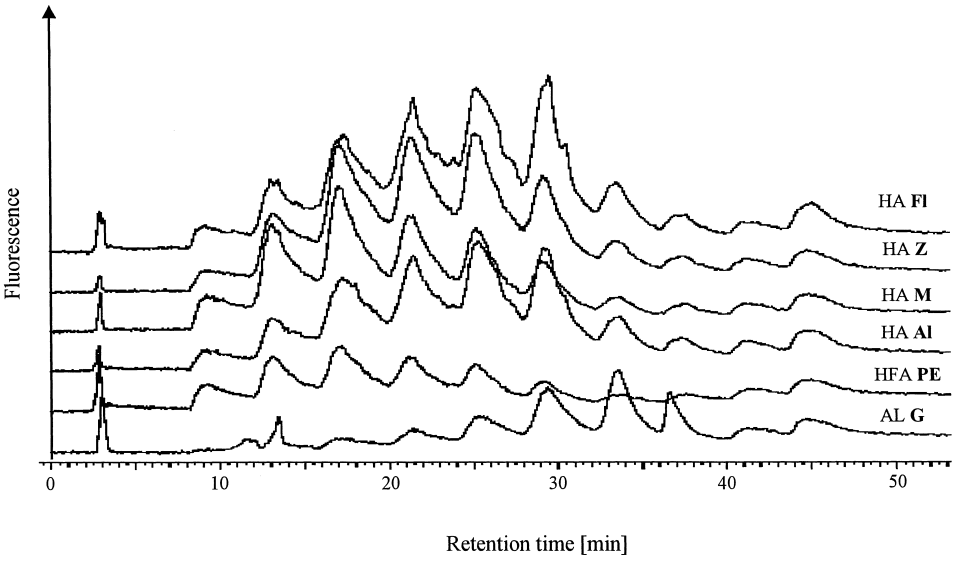

Fig. 5. Chromatographic profiles of various humic substances and alkalilignin as obtained by stepwise gradient elution at 1.00 ml/min detected by fluorescence detector (Ex. 470 nm, Em. 530 nm). Abbreviations: AL G, alkalilignin; HFA PE, peat humic acid; HA Al, Aldrich humic acid; HA M, soil humic acid; HA Z, soil humic acid; HA Fl, Fluka humic acid. For the other conditions, see Section 2.

dialysis (membrane with MWCO 8000) desalting. Humic acid HA M was obtained directly from soil sample by the procedure described in detail [36], whereas humic acid HA Z was obtained from the solid rest of the same soil after extraction of the soil (by sodium pyrophosphate extractant) leading to the HA M. Humic acid HA Z was obtained by dissolution/precipitation procedure after its release from the soil matrix by acid hydrolysis (6 *M* HCl). Thus, these two humic acids are of the same origin. In Fig. 5, comparing chromatographic profiles HA M and HA Z we can see that the highest peak of humic acid HA M elutes at lower retention time (17.1 min) than the highest peak (25.4 min, the last one of three peaks of similar height) of humic acid HA Z. However, SEC profiles [51] of these two humic acids were almost identical, resulting in the same relative molecular masses (Table 1). We could conclude that the humic acid HA M is more hydrophilic than the humic acid HA Z, which is so tightly incorporated or bound to the soil matrix that it must be released by strong acid hydrolysis.

Characteristic profiles produced by the proposed method can be compared to the published ones, in spite of the fact that separation mechanism and actual used conditions can be different. Ion-pair RP-HPLC method of Smith and Warwick [9] for fractionation of FAs based on one step gradient of tetrabutylammonium in phosphate buffer and polymeric PLRP-S (30 nm pores) column gives six peaks profile. Four of these broad well resolved peaks were attributed to FAs with rough appearance of our profiles. However, in our case each step of 10 steps gradient produces just one broad peak of particular HS. Apex of the eluted peak is always shifted with respect to the gradient step boundary. In contrary, four step gradient elution (acetonitrile in sodium acetate buffer) of marine dissolved organic matter (DOM) fractions from conventional (8 nm) C_{18} silica column published by Lombardi et al. [15,16] produces narrow but severely tailing peaks, probably due to their partial or restricted access into the pores and their displacement by sharp step boundary of acetonitrile. Here, again, each step produces just one narrow peak. Profiles published by Lombardi and Jardim [17] comparing marine DOM and terrestrial soil FA are represented by broader peaks with superimposed spiky peaks. Appearance of our profiles with respect to HS's peak shape is similar to the profiles published by Woelki et al. [18] obtained by two step gradient of acetonitrile in water and wide pore (100 nm) RP column. They have clearly shown

importance of careful pore diameter selection for HSs resolution in RP-HPLC. However, detailed comparison of the published profiles with those discussed in this work is not possible correctly, because Woelki et al. [18] used beside the solvent gradient also two step flow-rate program and non-buffered mobile phase.

Separation process itself dilutes the HSs and so sensitive detection method with low LOD is preferred.

Fig. 6 shows background corrected chromatographic profiles of humic-like substances (chromatogram APHLS B2) extracted by NaOH solution from sampled air particulates. For comparison purpose also chromatogram obtained from soil humic acid HA Z was added, because the soil sample from which this HA was extracted resembles the soil and potentially air dust particles in the locality where air particulates were collected. Because of a lack of proper standard materials HA Z was chosen also as a calibration standard for quantitation of humic-like substances in air particulates. As is seen even for this type of sample (APHLS B2) our method enables to get reasonable data and aids fractionation of humic-like substances for further investigation.

3.3. Quantitative aspects

Recovery of the method as evaluated by spectrophotometry using DAD ranged for the individual HSs from 94% to 102% at concentration levels around 3 mg/ml.

Quantitative parameters of the method were tested during investigation of humic-like substances isolated from air particulates (including soil dust particles).

Attempts to determine or estimate concentration of humic-like substances were based on such carefully chosen assumptions, as are:

- Construction of calibration curves from solid HSs which were obtained from soil, sediment or peat sample originated from the same or close locality as is locality where air particulates were collected by well defined and reproducible procedure
- Similarity of chromatographic profiles of the HS used as the calibration working standard with the profile of analysed humic-like substances
- Selection of a proper peak from the profile for the quantification (the peak must have almost the same relative peak area for both sample and the standard)

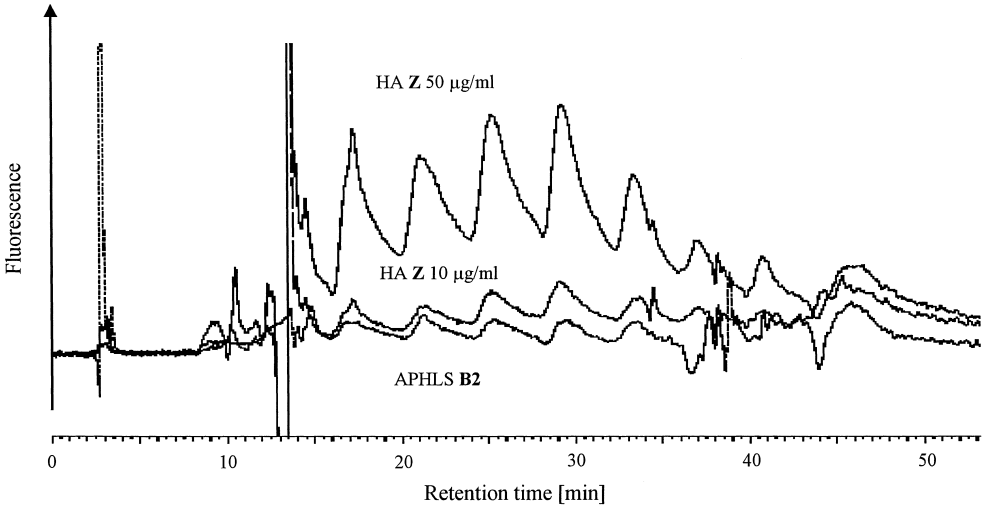

Fig. 6. Comparison of background corrected chromatographic profiles of air particulates humic-like substances (the lowest record APHLS B2) and soil humic acid HA Z (at concentration levels of 10 and 50 μg/ml, respectively) obtained by stepwise gradient elution at 1.00 ml/min and detected by fluorescence detector (Ex. 470 nm, Em. 530 nm). For the other conditions, see Section 2.

• Calibration measurements must be done at concentration levels that give signal close to the analyte signal.

Reproducibility of retention times (Table 2) of artificial peaks of HSs were evaluated from the data obtained in seven consecutive runs of sample HA Z at concentration level 3.2 mg/ml (at 420 nm, for the conditions see Fig. 4 and Fig. 2, respectively). Calculated reproducibility of retention time (t_R) of selected peaks is given in Table 2 and covers interval from ±0.12 to ±0.39% RSD. Decrease of RSD values with retention time increase is evident and can be attributed to constant standard deviation (SD) of time of individual steps formation (instrumental parameter) in a step gradient that results in a small values of SD of peak elution time related to beginning of particular gradient step eluting this peak. Thus, RSD related to SD of peak retention time calculated from point of injection (chromatographic parameter) is decreasing with the peak retention time increase. Repeatability of the data within 1 week (set of five data) was ±1.2% RSD. These data are representative also for the other well-shaped peaks of analysed substances.

Fig. 6 shows two calibration chromatograms (HA Z 50 μg/ml and HA Z 10 μg/ml) out of five used for the calibration curve construction in the range from 10 to 100 μg/ml. As a reference soil humic acid HA Z was chosen, because it fulfills the requirements defined above. The arable soil is of similar type as the soil in the field where the air particulates were sampled (Fig. 6, the lowest record APHLS B2).

Table 2
Retention reproducibility of selected HA Z peaks, linear regression values, and fitting coefficients

Peak no.	t_R	RSD [%]	A_1	A_0	R^2
3	16.91	±0.39	7.883e−005	−1.46	0.9683
4	21.13	±0.38	7.857e−005	−1.68	0.9923
5	25.22	±0.13	9.269e−005	−2.68	0.9808
6	29.14	±0.25	7.925e−005	−2.50	0.9687
7	33.61	±0.12	1.684e−004	−1.82	0.9894

t_R is average retention time in min; RSD is relative standard deviation of retention times calculated for seven runs.
Regression model was linear-response calibration function for five concentration levels: $Y = A_1 \cdot X + A_0$, where Y denotes analyte concentration in [μg/ml], X denotes peak area [μV·s], A_1 is calibration curve slope, A_0 is intersect, R^2 is variance of correlation coefficient.

Calibration curves (Table 2) were constructed for several peaks (having average retention times 16.91, 21.13, 25.22, 29.14, and 33.61 min) not disturbed by the residual irregularities of baseline remained after background correction or by narrow peaks of non-humic chemical individual present in the sample. Linear-response calibration function (HSM output) was chosen, what means that in linear model $Y = A_1X + A_0$ integrated peak areas (denoted as X) are processed versus concentration of injected sample HA Z (denoted as Y). The integration was done for chosen peaks and baseline was set from valley to valley. For the fluorescence detection evaluated calibration curves were linear for the selected peaks with correlation coefficients of linear regression (least-squares method) between 0.9840 and 0.9961 (from data in Table 2).

Quantitation of humic-like substances in air particulates was based on individual calibration curve for the peak having retention time 21.13 min and regression equation $Y = 7.857 \times 10^{-5}X - 1.684$. Further information on statistical treatment of the equation was not available in the HSM software, except the correlation coefficient 0.9961.

This peak was selected according to criterion that it has almost the same relative peak area (13.7±1.6%) for working standard HS Z, as has corresponding peak (retention time 21.15 min) of humic-like substances B2 in air particulates (13.9%). Relative peak area of HS Z was statistically evaluated from chromatograms measured at five concentration levels) and was related to the total of the areas of all the components detected in the chromatogram (area% method). Similarly we got the value for humic-like substance APHLS B2.

Determined concentration was 9.7 μg/ml, what corresponds to the limit of quantitation ($S/N = 10$) of the method with fluorimetric detection. This corresponds roughly to 0.5 μg of HS per injection. Calculated limit of detection ($S/N = 3$, from chromatogram without background correction) was 3.3 μg/ml, what corresponds to 0.15 μg of humic-like substance APHLS B2 per injection.

After recalculation to the humic-like substance

concentration in the air particulates we got value 0.97 mg/g, what corresponds to relative mass concentration close to 0.1%; however, expressed and interpreted as a concentration of similar soil humic acid (in our particular case HA Z), chosen arbitrarily as a working standard according to the defined rules.

Described method produces multidimensional data of HSs (chromatographic, UV–Vis spectra, fluorescence) which can be processed by methods of chemometry (e.g. pattern recognition, clustering etc.), or for transformation of HS chromatogram, for instance to elution profile of aromaticity $Q_{4/6}$ (ratio of absorbances measured at 465 and 665 nm). However, these aspects of data processing are outside the scope of this article and will be published elsewhere.

4. Conclusion

The results suggest that the devised method is highly reliable for characterisation and fractionation of terrestrial HSs, air particulate humic-like substances and even lignins in a wide concentration range and also at trace concentration levels. Obvious differences in their profiles, as well as quantitative parameters, beside highly polar character of DMF designate this stepwise gradient HPLC method using wide pore RP bonded phase for its combination with other HSs characterisation methods, preferably separation methods. Individual fractions obtained by the described RP-HPLC method can be analysed by the methods working on independent principles, e.g. capillary isotachophoresis (CITP) [38,39], capillary zone electrophoresis (CZE) [42,43,46], on-line CZE-ITP [44,45] or SEC [29] in a similar way as was already published by Hutta et al. [40] and so produce data of even higher dimensionality needed for more detailed HSs investigation.

The sensitivity of the method enables also further characterisation of fractions of HSs collected from independent (preferably orthogonal) separation method, e.g. micropreparative CITP [41].

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